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Scanning for point variation in large populations by melt-MADGE

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▼*De novo* detection of base substitutions in amplimers is not possible without exploiting natural or inducible conformational differences in some way. There are now many techniques of conformation-dependent, rather than size-dependent, electrophoretic separation. The most popular, single strand conformation polymorphism (SSCP) analysis (Ref. 1), detects the sequence-specific folding of single stands, which depends on chance internal base pairing. However, there is no theory to predict whether or how a single strand will interrogate all its own internal bases and what mobility it will develop in a given electrophoresis. Other techniques use chemical or enzymatic cleavage of mismatches, modified acrylamides or mild denaturants to detect heteroduplex moieties formed during PCR with heterozygote templates [heteroduplex analysis or conformation-sensitive gel electrophoresis (CSGE) (Ref. 2)]. These adequately detect heterozygosity but, for mutant homozygotes to be detected, an extra step of co-annealing with one of the alleles and a second electrophoresis are required.

Reconfiguring the above techniques for microplate-array diagonal-gel electrophoresis (MADGE) should be straightforward. However, we favoured and developed a melting-point analysis because this is theoretically 100% sensitive to point and frameshift variations, and is backed by a precise mathematical model that predicts the electrophoretic behaviour and resolution of alleles along a temperature (or denaturant concentration) gradient. A melting-point analysis by MADGE [melt-MADGE (Ref. 3, 4; E. Spanakis *et al.*, unpublished)] can thus be designed by computer and offers unprecedented throughput for *de novo* mutation scanning with a simple and inexpensive set-up.

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1. The principles and current limitations of melting-point analysis

The theory of DNA melting (Ref. 5) is the basis of four established scanning techniques. The probability of dissociation of each base pair along a DNA sequence (its melting profile) depends on the number of hydrogen bonds holding the bases together and on the adjacent base pairs (Ref. 6). Most point and frameshift mutations alter the melting profile of the domain in which they occur. Partial dissociation of the strands results in a proportional increase of their effective length, but an exponential electrophoretic retardation, of a DNA molecule.

Conservative transversions that do not alter the number of hydrogen bonds between the strands might, in some sequence contexts, produce no measurable thermodynamic effects. Even these mutations can be detected when in a heterozygous context by the appearance of heteroduplex bands. The thermodynamic effects of mismatches and 'bubbles' (mismatches owing to small insertions or deletions) are always greater than those of loss of a hydrogen bond and are readily detectable.

In the classical but laborious denaturant-gradient gel electrophoresis (DGGE) technique, allelic molecules run against increasing concentrations of a chemical denaturant (usually urea and formamide) at a constant temperature. Differences in the retardation and resolution of alleles occur only within a narrow zone of the gradient, in which the most thermostable allele remains double stranded and moves rapidly while alleles with lower melting points unwind and slow to various degrees. The rest of the time taken to run the gel (typically overnight) is wasted, and so is most of the gel, because, before and after dissociation, all alleles have equal velocities. Resolution is inversely proportional to the slope of the denaturant gradient.

A simpler version, constant-denaturant gel electrophoresis (CDGE), gains time using the effective denaturant concentration in chemically homogeneous gels running at

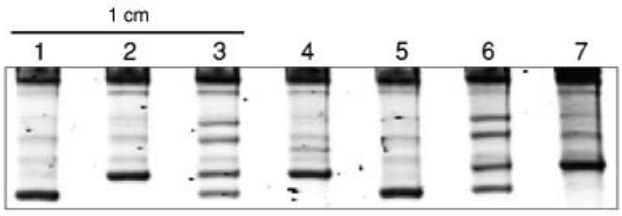

Fig. 1. Allelic amplimers of the 3′ UTR of the human BRCA1 gene that are resolved by 6 mm of electrophoresis; long tracks are not necessary. Fast-allele homozygotes are in lanes 1 and 5, slow-allele homozygotes in lanes 2, 4 and 7 and heterozygotes presenting two homoduplex and two heteroduplex bands in lanes 3 and 6. The real width of the tracks is 2 mm.

a constant temperature. Alleles adopt different velocities from the outset and resolution is proportional to run time. In loci with complex melting behaviour, however, CDGE can miss variations because the precise chemical and thermal conditions used define only a narrow range of melting points that can be resolved (Ref. 7, 8).

The latest version of melting-point analysis by electrophoresis, temporal temperature gradient electrophoresis (TTGE; Ref. 9), retains the convenience of homogeneous denaturant gels and compensates for the lost sensitivity by varying the gel's temperature over time (temperature ramps). Denaturing HPLC replaces electrophoresis with automated denaturing chromatography but still requires an overnight run to complete the analysis of 96 PCR products (Ref. 10).

The above electrophoresis techniques traditionally use vertical gels offering long tracks for detection of small mobility differences at the expense of throughput. However, partial dissociation of the double helix introduces large differences in mobility and long tracks, or runs, are not needed (Figs. 1 and 2).

2. Amplimer (and primer) design

The *Melt87* algorithm (Ref. 5) computes the melting profile of the sequence to be scanned. We have implemented *Melt87* and extended its algebra substantially in a Visual Basic package that takes advantage of the text editing and graphical capabilities of Microsoft *Word* and *Excel* (E. Spanakis, unpublished). (This program is available from the authors at the University of Southampton.) Alternative commercial applications are available from MedProbe (Oslo, Norway) (http://www.medprobe.com/xx/melt.html).

Amplimers are designed according to the same principles as for DGGE, CDGE or TGGE. To avoid complete dissociation of the strands, and thus to maximize the electrophoretic retardation achieved by partial melting, the variable sequence needs to be 'clamped' with a small GC-rich thermostable domain [11]. Clamps can be added by PCR using a GC-rich extension in one of the primers. The following extension has been repeatedly tested and can be used as a universal clamp at either end.

5′-cgcggcggagcgaggcccgcgggcccgcccgccgcgcccc-3′

Slightly sloped melting profiles (i.e. melting occurs over a range of temperatures) are flattened by clamping the thermolabile end. We frequently clamp both ends, the second with a much smaller clamp (5–20 G/C) to obtain perfectly flat melting domains. Any number of nucleotides from the 5′ end of above clamp sequence can be used, in the same order, to flatten the melting profile at the other end if necessary. Flat melting profiles are convenient because variation can be detected along the entire length of a domain, with maximal resolution, at a constant temperature (using CDGE).

More complex melting profiles require a temperature ramp or a more complex temperature profile during electrophoresis. As the complexity of thermodynamics increases, the theoretically achievable resolution decreases and mutations might be missed. Also, CDGE has a lower requirement for apparatus than thermal ramping because the temperature must simply be held constant, rather than varied in a controlled fashion. The limitations on mutation detection related to complex melting profiles or clumsy amplimer design are not specific to melt-MADGE but are inherent in all melting-point analysis techniques, including DGGE, CDGE, TTGE and DHPLC.

Primers with CG-rich extensions at their 5′ end also help PCR. Never use computer programs that select PCR primers automatically. Unless explicitly stated otherwise, their criteria are completely irrelevant to melting-point analysis and the amplimers so designed might be inadequate for mutation detection. However, programs that check for folding and dimer formation can be consulted. A certain degree of innocuous folding and dimerization is expected, particularly with GC-rich sequences, owing to the low complexity of the clamp sequence. In our experience, much more attention should be given to the melting profile of the amplimer than to designing perfect primers with traditional criteria. Obviously, sequences that are designated as repeats in a Genbank record and sequences with visibly low complexity (homopolymeric tracts, microsatellites etc.) should be strictly avoided.

3. PCR

Basic PCR chemistry, cycling and optimization principles apply, but clamped primers make the reaction much more

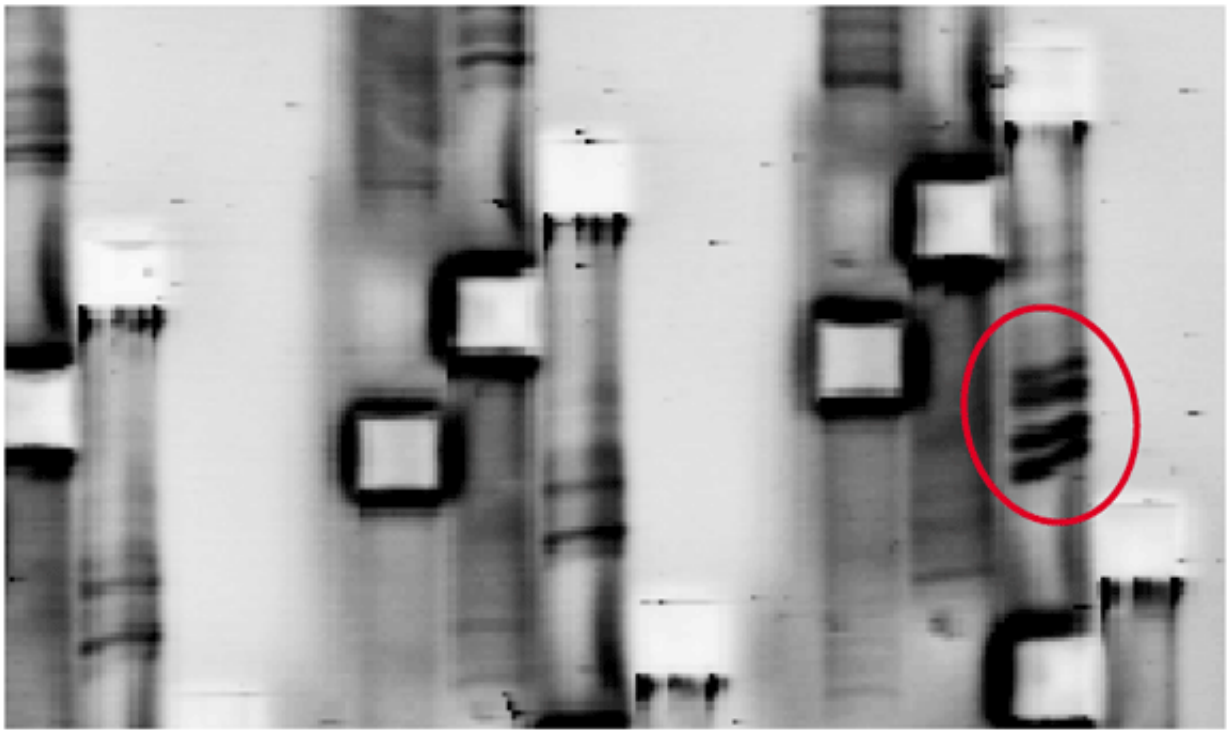

Fig. 2. Magnification of a high-density MADGE gel (384 wells, 1 cm track length) in which a heterozygote with four strong bands (the circled sample) is readily distinguished from homozygotes. Melt-MADGE384 is particularly useful for *de novo* scanning for rare mutations. The capacity of a melt-MADGE tank is 12×84 1-cm tracks and electrophoresis takes only 30 min.

robust than common 20mers do and tight optimization is not usually necessary. We typically use 0.3 units of standard Taq DNA polymerase (GibcoBRL Life Technologies, Paisley, UK) for 20 ml oil-free reaction mixtures containing 10 mM Tris (pH 8.3), 50 mM KCl, 0.01% w/v gelatine, 1.5 or 3 mM $MgCl_2$, 20 nM template DNA, 16 pmol of each primer and 200 μM dATP, dCTP, TTP and dGTP. Some sequences might require the addition of DMSO (e.g. 0.5% v/v).

A single cycling program of 96°C for 2 min followed by 30 cycles of 96°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and finishing with 72°C for 2 min will work most of the time [running on an NJ Research DNA Engine™ Tetrad series thermal cycler (Genetic Research Instrumentation Ltd, Rayne, UK)]. Optionally, final steps at 96°C for 5 min and 85°C for 5 min might encourage the formation of heteroduplexes. For sequences with melting points well above 72°C, annealing and extension can be combined into a single step at 72°C for 1 min.

Gel

The denaturing gel mixture consists of:

5–6% acrylamide:bisacrylamide (depending on the size of the amplimer – a 445 bp amplimer required 5% acrylamide but shorter amplimers resolved at either concentration);
4–8 M urea (depending on the melting point of the sequence assayed);
1× TAE (40 mM Tris, 1 mM EDTA, brought to pH 8 with glacial acetic acid).

We use a 19:1 acrylamide:bis acrylamide stock solution (Severn Biotech, Kidderminster, UK). Other ratios might theoretically improve the resolution of some sequences but we have not yet needed to optimize the gel matrix. The quality of the stock acrylamide solution and of the urea and APS are critical, and the use of fresh (daily) solutions of urea and APS is strongly recommended.

Deviation from the above composition is possible. For example: the buffer concentration may vary at least between 0.75× and 2×; TAE may be replaced with TBE; and urea may be dropped below 4 M for AT-rich sequences. However, the temperature and duration of electrophoresis will have to be readjusted empirically with every change in the gel composition. In general, identical results can be achieved with

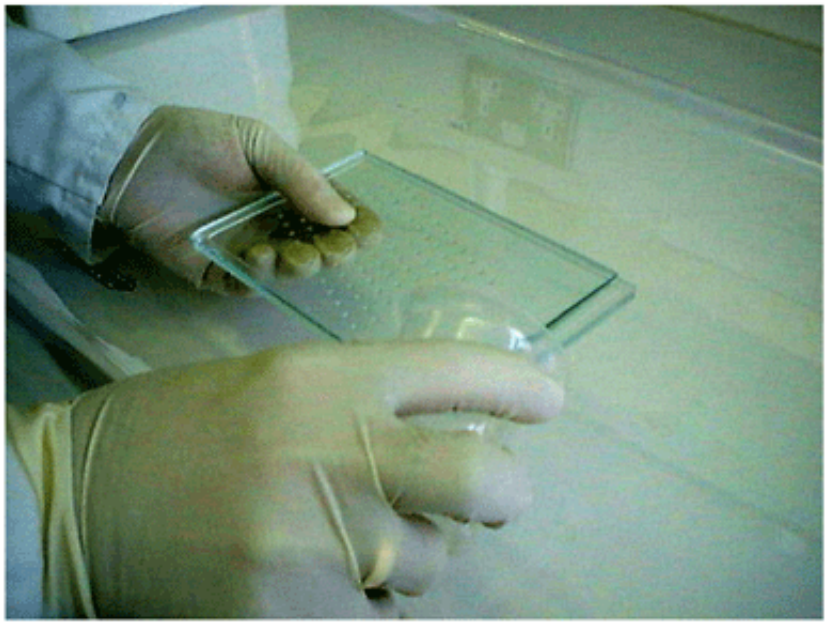

Fig. 3. There are several ways to cast a gel. Here, the gel-former and the glass plate are held together and the gel is poured in the space between them. The operation takes seconds and no special preparation or assembly is needed.

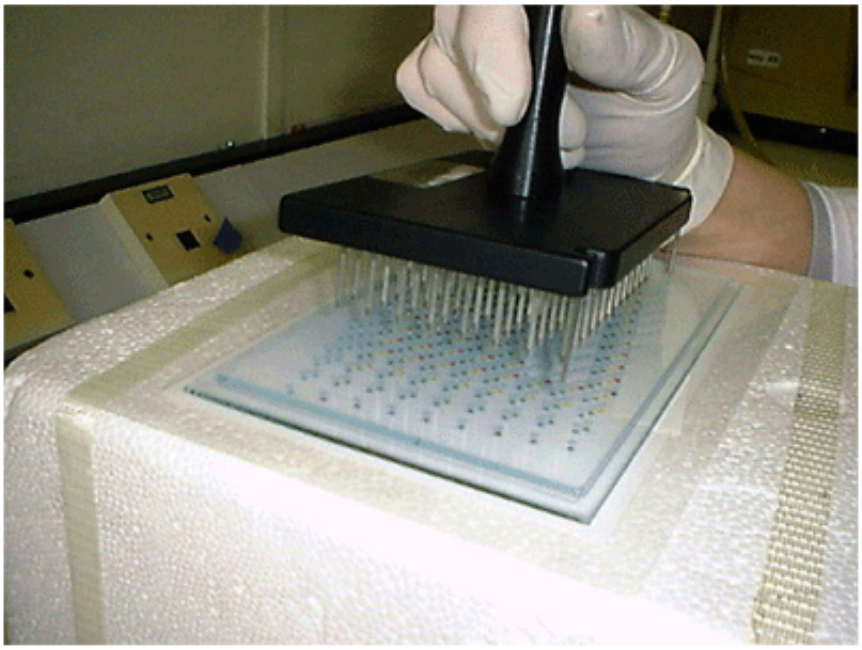

Fig. 4. We use a passive replicator to load 96 wells at once directly from a PCR microplate.

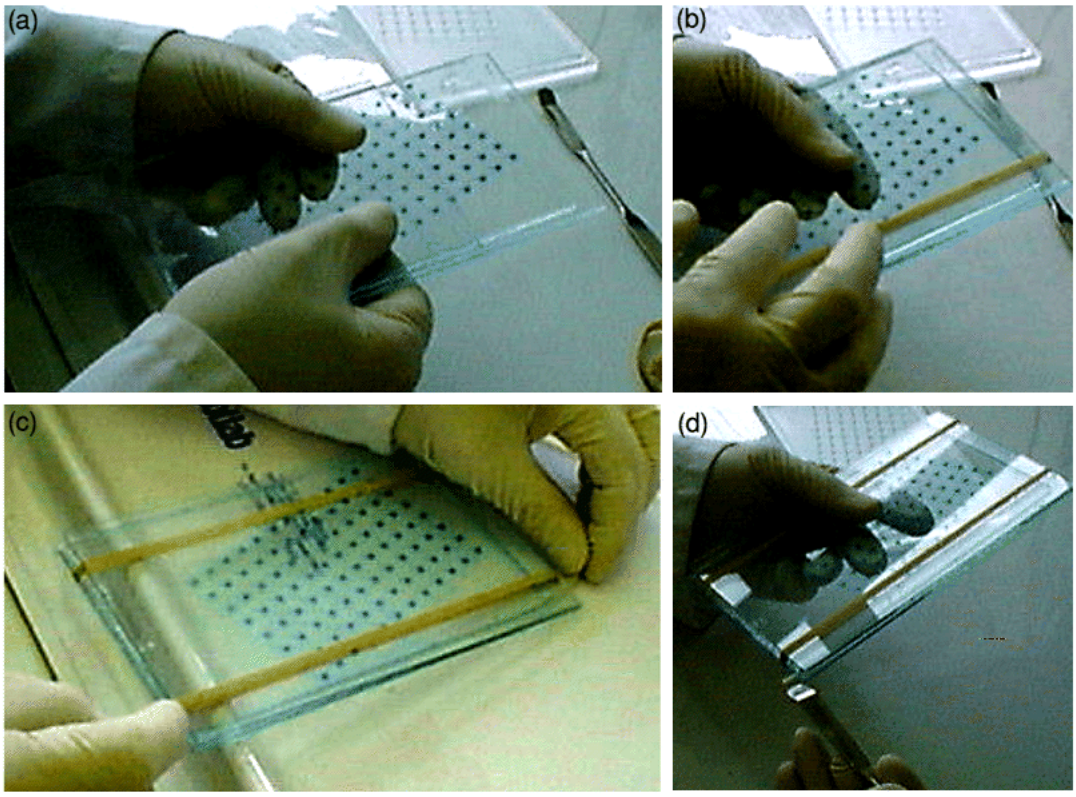

Fig. 5. After loading, the gel is covered with a second glass plate (a). The glass–gel–glass 'sandwich' is secured with two stationary rubber bands (b). The long edges of the gel are sealed with ~15cm-long pieces of silicon rubber tubing forced between the glasses to touch the gel (c,d).

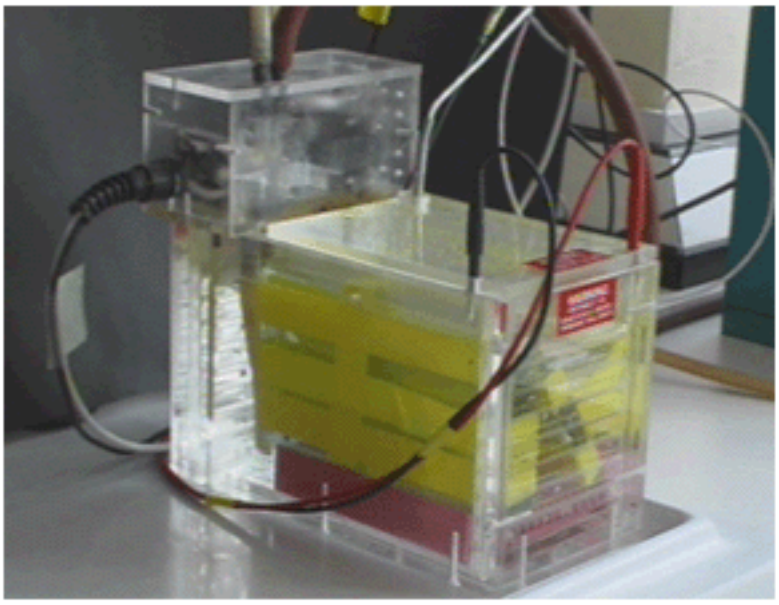

Fig. 6. A simple, home-made, cuboid electrophoresis tank with 2 l capacity accomodates up to 12 gels.

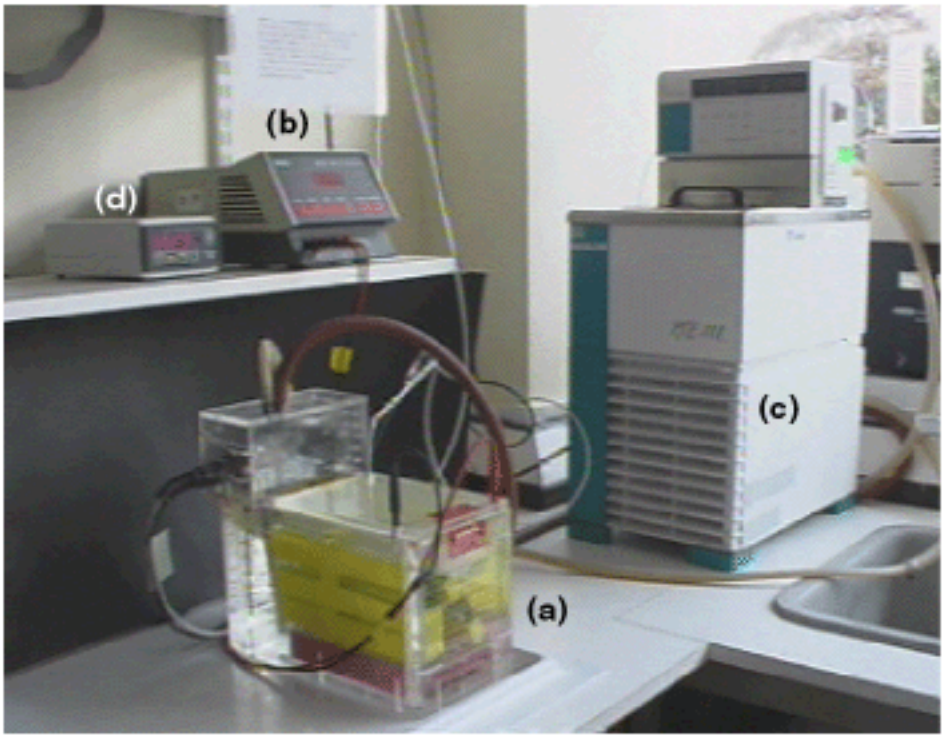

Fig. 7. The tank (a) is connected to a commercial power supply (b) delivering up to 200 V and 2 A, and to a commercial cooler–circulator (c).

quite different sets of conditions, which means that the system is versatile and robust. Formamide, used as an additional denaturant in DGGE, CDGE and TTGE assays, is not suitable for melt-MADGE.

Gel casting and stting

Gel casting takes only seconds. A MADGE former and a glass plate with the same dimensions are required.

Wipe one surface of the glass plate with sticky silane (0.5% v/v γ-methacryloxypropyltrimethoxy silane and 0.5% v/v glacial acetic acid in ethanol).
Hold the glass with the silanized surface against the gel former.
Initiate polymerization with 2 mM APS (final) and 0.2% v/v TEMED.
Without delay, pour the gel into the gap between the former and the glass (Fig. 3).
Let the gel set for ~30 min.

With the above concentrations of ammonium persulfate (APS) and TEMED, visible polymerization occurs within 4–5 min and the gel is robust and can be lifted within ~10 min. The gel-setting time can be cut down to 10 min or extended up to 60 min. For reproducibility of results between experiments, however, approximately the same gel-setting time should be respected.

Loading

Use a multichannel loading device (Fig. 4) for convenience and speed (they are all compatible with PCR microplates and with MADGE). No loading buffer is required but some dye (e.g. traces of bromophenol blue) can be added to the PCR products to improve visibility.

Load exactly 8 μl of (diluted) PCR product per well.
Cover the gel by sliding a clean glass plate over it (Fig. 5a).
Secure the assembly with two rubber bands (Fig. 5b).
Seal the long edges of the gel with two 15 cm pieces of 2-mm-diameter silicone-rubber tubing inserted between the glass plates [stretch, insert and release (Fig. 5c), then push until the tubing touches the gel (Fig. 5d)].

A 96-split-pin replicator (V&P Scientific, San Diego, CA, USA) loads 2 μl at a time, which is sufficient for most PCR reactions. Larger volumes can be loaded with repeated transfers or with a pressure-driven replicator [e.g. simultaneous plate-loading and transfer tool (SplatT) (Intelligent Bio-Instruments, Cambridge, MA, USA)]. If the volume of the samples is less than 8 μl, complete with water; a few

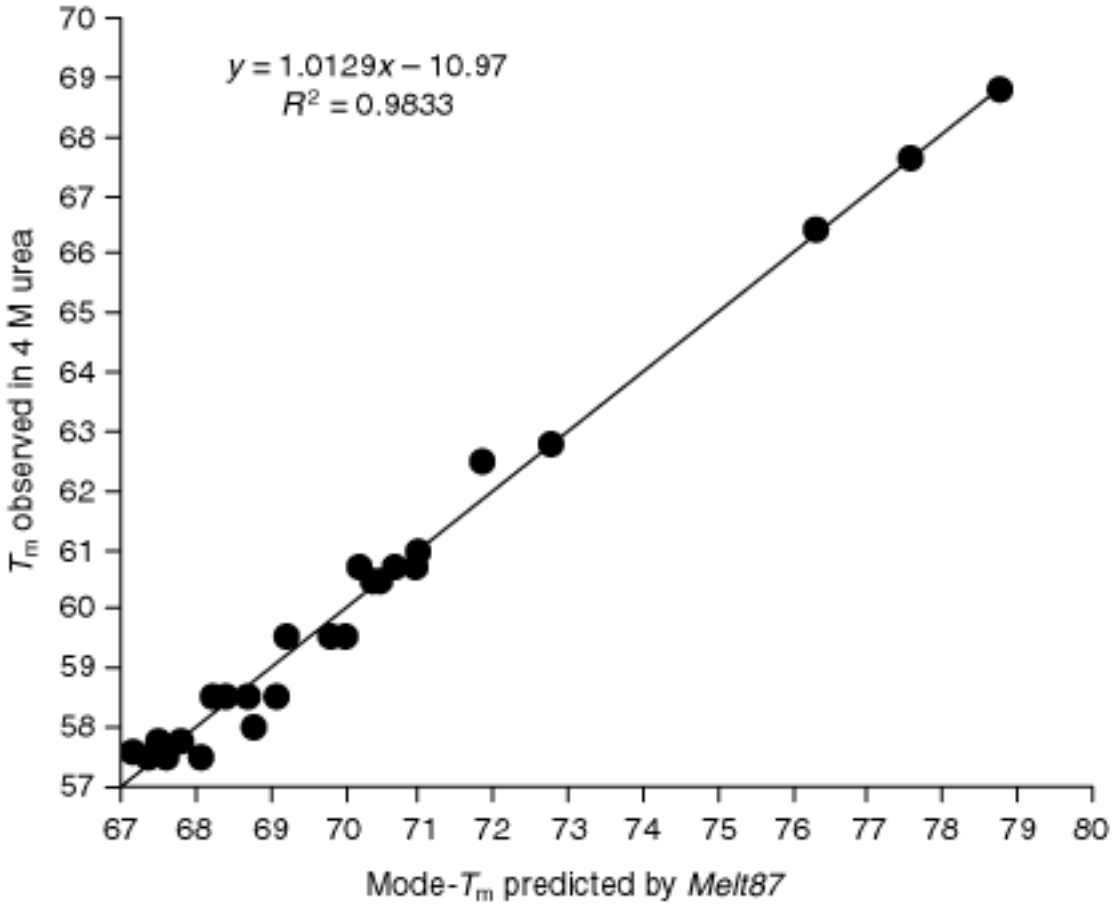

Fig. 8. The correlation between T_m values predicted by the *Melt87* algorithm (Ref. 5) and the temperature at which amplimers melt during melt-MADGE with 4 M urea is almost perfect; more than 98% of the variance is explained by linear regression. The intercept of regression indicates that 1 M urea reduces the predicted melting point of a sequence by ~2.7°C. The statistics are based on theoretical and experimental analysis of 35 *BRCA1* amplimers.

drops placed on the surface of the gel will fill up the wells during covering.

Any air bubbles trapped in under-loaded wells will severely affect band definition in the corresponding tracks. However, never lift the cover glass to release trapped air as this will remove all samples from their wells and mix them up. Loading more than 8 μl might cause visible well-to-well contamination but, when all PCR products have approximately equal concentrations, such contamination does not impair interpretation because contaminant bands are much fainter than real ones.

Electrophoresis

A small electrophoresis tank under good temperature control is required. Our prototype tank (Figs. 6 and 7a) is 23 cm long (anode to cathode), 11 cm wide and 15 cm high, with a 2 l maximum capacity. It has two platinum electrodes, a propeller, a water-circulating glass coil and a removable gel rack. The tank is made of 0.5-cm-thick polypropylene. The electrodes are connected through the cover of the tank to a commercial 200 V, 1 A power supply (Fig. 7b). Perfect spatial homogeneity is achieved by vigorous stirring. The glass coil is connected to a programmable heating–cooling circulator (Fig. 7c) that can produce a linear temperature ramp up to 70.00°C [e.g. RT111, Neslab, (http://www.neslabinc.com/), Runcorn, UK]. An apparatus with built-in temperature control would offer a more compact, but dedicated, set-up.

The performance of prototype equipment should be monitored with a high-precision (±0.01°C) digital thermometer (Fig. 7d) calibrated back to national standards [e.g. Tracker 120 (Data Track Instruments, New Milton, UK); calibrated by Universal Calibration Laboratories (Romsey, UK)]. The rack, also made of polypropylene, could accommodate

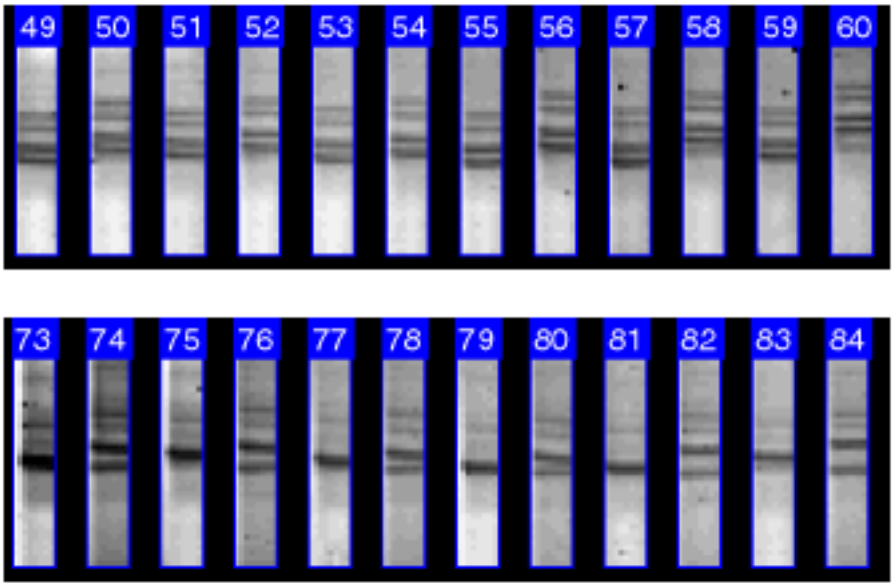

Fig. 9. Track alignment by Phoretix software can be used for column-wise comparisons of patterns. In this example, model homozygotes and heterozygotes for the common alleles $\varepsilon 2$ (TT haplotype), $\varepsilon 3$ (CT haplotype) and $\varepsilon 4$ (CC haplotype) of human *APOE* exon 4 were loaded alternately at various positions of a melt-MADGE to show that the gel offers the same resolution throughout its surface from anode to cathode. (a) The $\varepsilon 3/\varepsilon 4$ pattern with intermediate-velocity ($\varepsilon 3$) and fast [$\varepsilon 2/\varepsilon 3$ patterns with intermediate-velocity and slow ($\varepsilon 2$) bands (even-numbered tracks). (b) The $\varepsilon 3$ band of a homozygote runs with intermediate velocity (odd-numbered tracks) compared with $\varepsilon 2$ and $\varepsilon 4$ bands of an $\varepsilon 2/\varepsilon 4$ heterozygote (even-numbered tracks).

12 gels, leaving a 2-mm gap between gels to allow free circulation of buffer and uniform distribution of heat through the stack.

Melt-MADGE requires a continuous buffer system. It is essential that the ionic environments of the tank and the gel be identical. For this reason, not only do we use the same buffer at the same concentration in the tank as for the gel but we also add to it the same final concentration of APS.

Tank buffer: 1× TAE, 2 mM APS.

The temperature of electrophoresis depends on the melting profile of the sequence and the concentration of urea in the gel (Fig. 8); 1 M urea reduces the melting point by ~2.5°C.

Typically, run at 100–200 V for 20–45 min, depending on the length of the amplimer and the voltage.

For reproducible electrophoretic velocities between runs, the total volume of the tank and gel buffer should not change.

Staining, imaging and pattern analysis

Once removed from the tank a melt-MADGE gel can be treated like any other polyacrylamide gel.

Stain with ethidium bromide or, preferably, a more sensitive fluorescent stain such as SYBR Gold or VistraGreen (Molecular Probes, Eugene, OR, USA).

Image with Polaroid photography or a CCD camera, using a fluorimager [e.g. model 595 (Molecular Dynamics Amersham Pharmacia Biotech, Little Chalfont, UK)], which offers greater versatility, colour range and imaging resolution, or another digital imaging device.

MADGE image analysis software is available from Phoretix International (Newcastle-Upon-Tyne, UK; http://www.phoretix.com/index2.html). The Phoretix software includes track alignment (Fig. 9) and image analysis tools for very fine migration and band-intensity measurements (Fig. 10). Semiautomatic genotype calling is also possible. In the first example (Fig. 9), a T > C heterozygote is compared with C > T heterozygote, loaded on odd and even tracks, respectively (tracks 49–60). Also, a CT-haplotype homozygote is compared with a CC/TT heterozygote, loaded on odd and even tracks, respectively (tracks 73–84). Another example of a full-size melt-MADGE image (Fig. 11a) with its corresponding aligned images (Fig. 11b) is also shown.

This peer-reviewed article can be cited as: Spanakis, E. *et al.* (2001) Scanning for point variation in large populations by melt-MADGE. *Technical Tips Online* P02204.

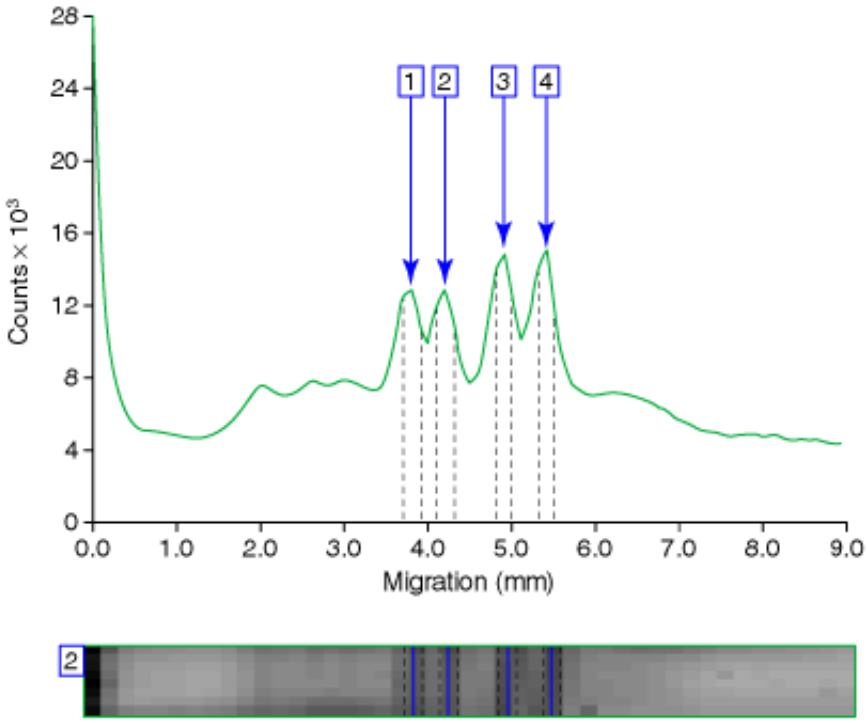

Fig. 10. The trained eye can recognize band patterns at a glance but image analysis can help in case of doubt. Phoretix was used here to measure precisely migration (*x* axis) and to quantify band intensities (*y* axis). In this example (APOE exon 4, $\varepsilon 2/\varepsilon 3$ heterozygote) the homoduplex bands 3 and 4 have equal intensities and the the heteroduplex, bands 1 and 2 are equally under-represented. A heterozygote pattern requires no more than 2 mm to develop in full and the run does not need to be longer than 5 mm.

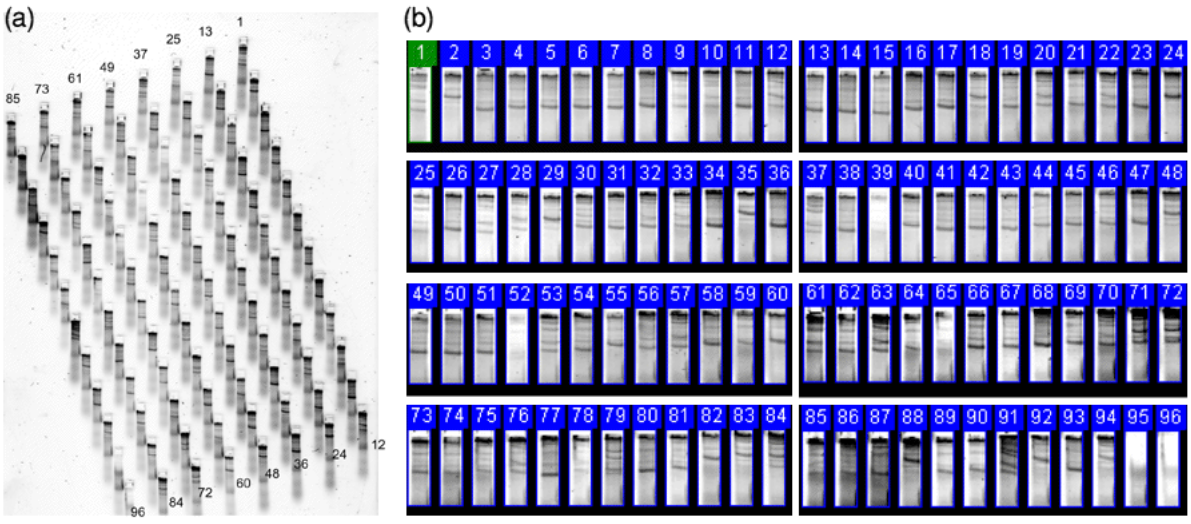

Fig. 11. A full meltMADGE gel displaying a novel, unidentified polymorphism around the poly-A signal of the *BRCA1* gene. Within-row comparisons of patterns can be made on the original image (a); within-column comparisons (from anode to cathode) are easier with the tracks aligned (b). Black arrows indicate homozygotes for the minor, slow allele and white arrows indicate heterozygotes. Tracks 95 and 96 contained PCR products with no template DNA and served as contamination controls.

Acknowledgements

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Products Used

Taq DNA Polymerase: Taq DNA Polymerase from Roche Molecular Biochemicals
Taq polymerase: Taq polymerase from Pharmacia
Taq polymerase: Taq polymerase from Bioline
Taq polymerase: Taq polymerase from Roche Molecular Biochemicals
Taq polymerase: Taq polymerase from Roche Molecular Biochemicals
Taq polymerase: Taq polymerase from Bioline
Taq DNA polymerase: Taq DNA polymerase from PE Applied Biosystems
Taq DNA Polymerase: Taq DNA Polymerase from Roche Molecular Biochemicals
Taq DNA polymerase: Taq DNA polymerase from Life Technologies (Gibco BRL)
Taq DNA polymerase: Taq DNA polymerase from Promega Corporation
Taq DNA polymerase: Taq DNA polymerase from Stratagene
Taq DNA polymerase: Taq DNA polymerase from Takara Shuzo
Taq DNA polymerase: Taq DNA polymerase from Amersham Pharmacia Biotech
Taq DNA polymerase: Taq DNA polymerase from Boehringer Mannheim
Thermal Cycler: Thermal Cycler from Techne (Cambridge) Ltd
thermal cycler: thermal cycler from MJ Research Inc
Acrylamide: Acrylamide from Sigma
19:1 acrylamide: 19:1 acrylamide from Severn Biotech
96-split-pin replicator: 96-split-pin replicator from V & P Scientific
SYBR Gold: SYBR Gold from Molecular Probes
VistraGreen: VistraGreen from Molecular Probes